Evolution of Syndromic approach for early infectious disease diagnosis and control

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Director Medical Office Purehealth

15:45: Thursday, 24 October 2019

Objectives

- Introduction
- Evolution of the Syndromic Approach to diagnosis
- Overview of Mol Biol in Disease diagnostics
- PCR
  - Multiples PCR & Gel based approaches
  - Real time PCR based approaches
  - Film / Bio Arrays
- Syndromic Approach to diagnosis using Sequencing
  - First Gen sequencing
  - Second Gen Sequencing or NGS technology (ES and WGS)
  - Fourth Gen Sequencing: Long read Sequencing
- References

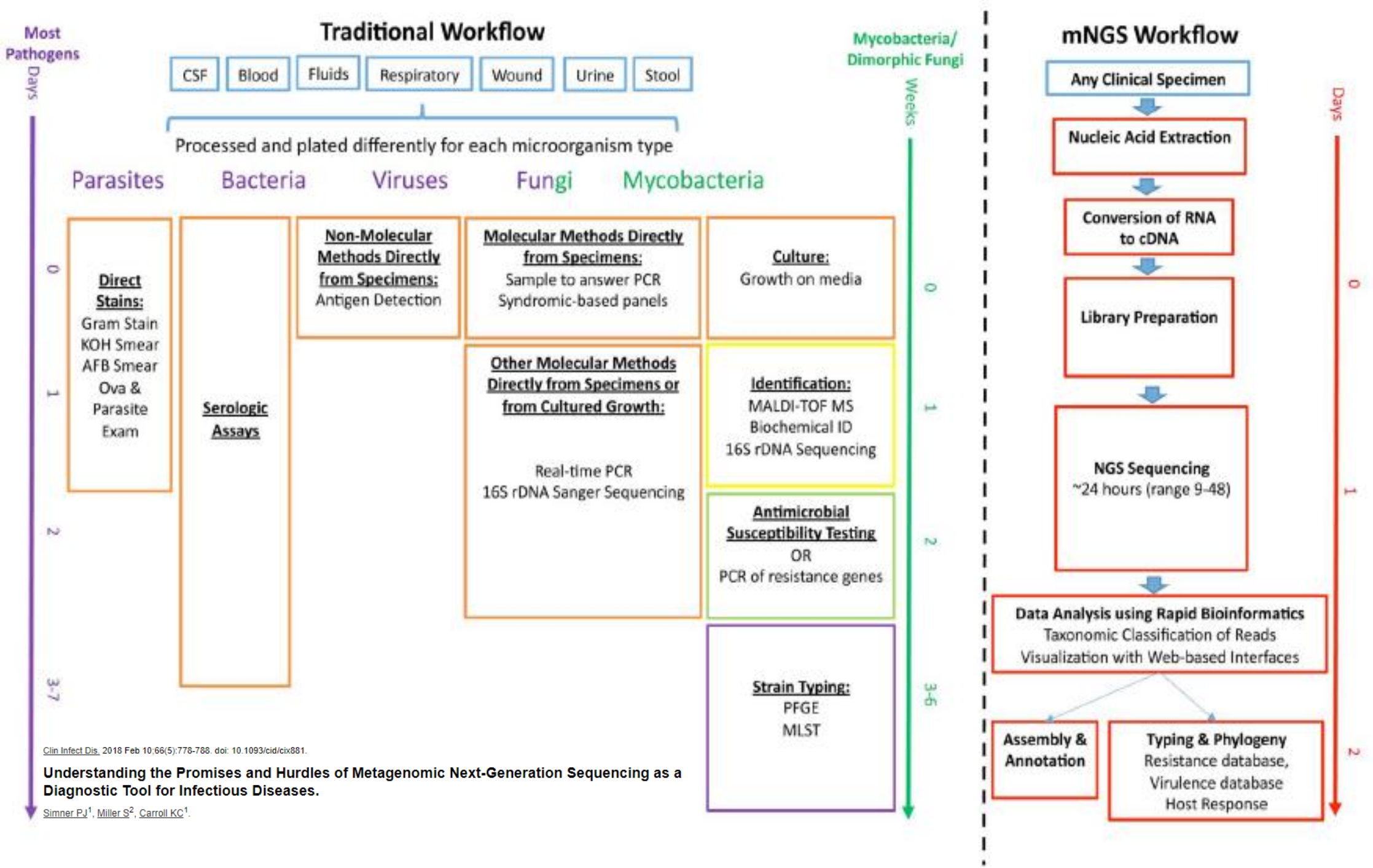
Introduction

Overview of Molecular Methods in the Diagnosis of Infectious Disease

Diagnosis of Known Pathogens	Diagnosis of known pathogens with unknown mutations	Emerging Technologies
ASO-probe Methods	Denaturing Gradient Gel Electrophoresis (DGGE)	Microarray Analysis.
PCR and ARMs-PCR	Sanger Sequencing	Next Generation Sequencing (NGS)
Restriction Enzyme Analysis	Multiplex Ligation- Dependent Probe Amplification (MLPA)	Third Gen Sequencing
Gap-PCR	Sequencing	Fourth Gen Sequencing
Real Time PCR	High Resolution Melting Curve Analysis (HRMA)	
Pyrosequencing		

ASO: Allele specific oligonucleotides, ARMs: Amplification refractory mutation system.

[Old J, Prevention of Thalassemias and Other Haemoglobin Disorders, 2nd ed. Cyprus: Thalassemia International Federation;2012].

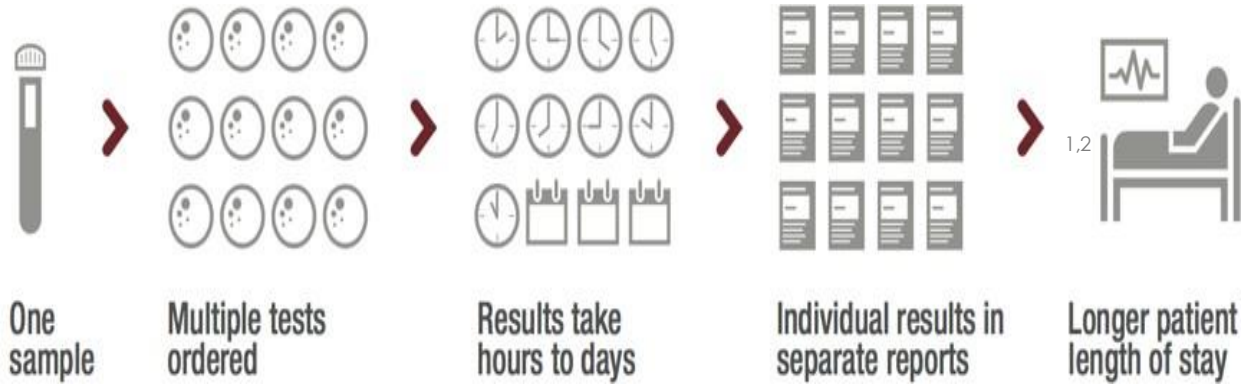


Futuristic

Fourth generation sequencing: nanopores:

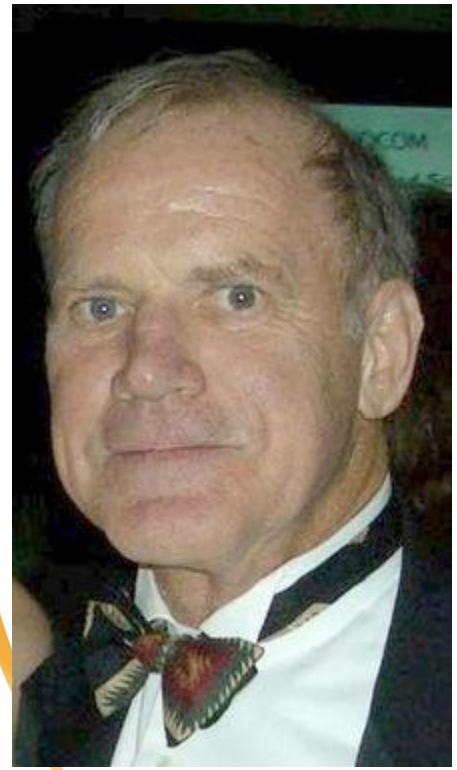
Traditional vs. Syndromic Testing

Traditional Testing



Syndromic Testing

PCR: for AMPLIFICATION OF DNA

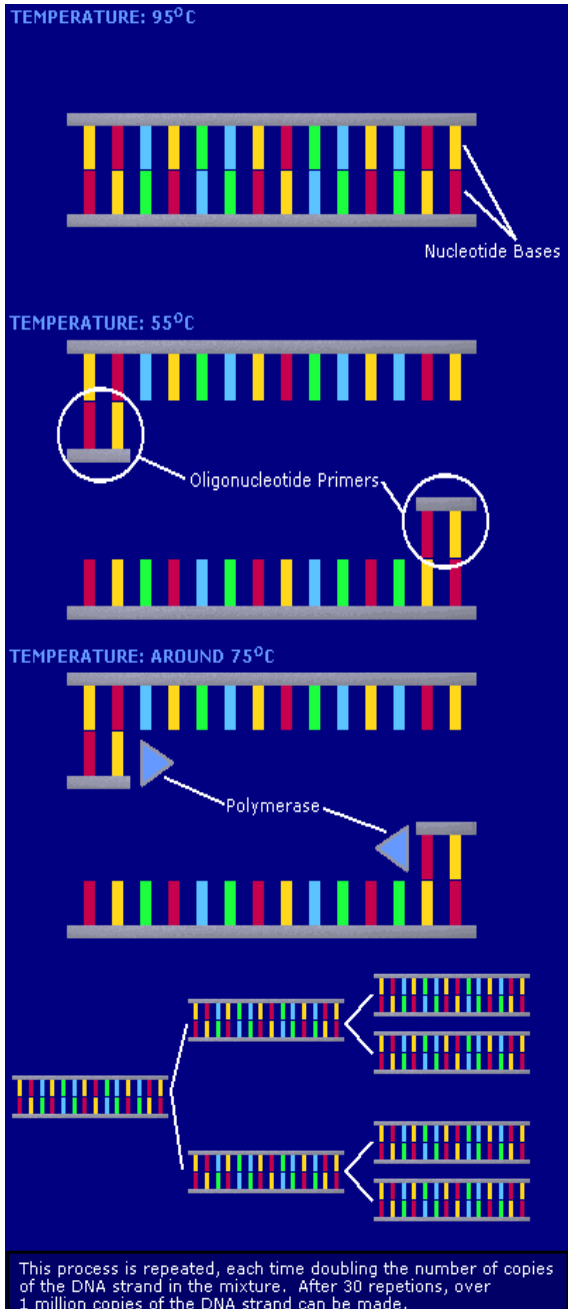


Kary Mullis
1944….Aug 2019

Denaturation

Annealing

Extension

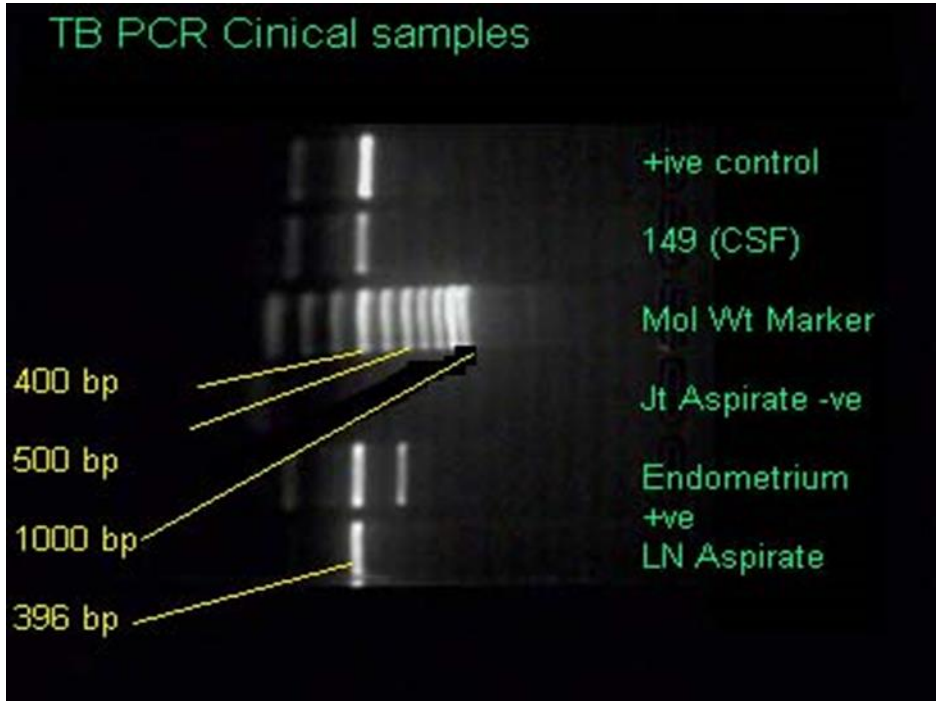


- Under appropriate experimental conditions the DNA molecule can be repeatedly replicated
- *Kary Mullis received the Nobel Prize in 1983 for discovering PCR technology*
- The Polymerase chain reaction (PCR) can be used effectively for diagnosis of infectious disease

One gene one Bacterium detection: TB PCR

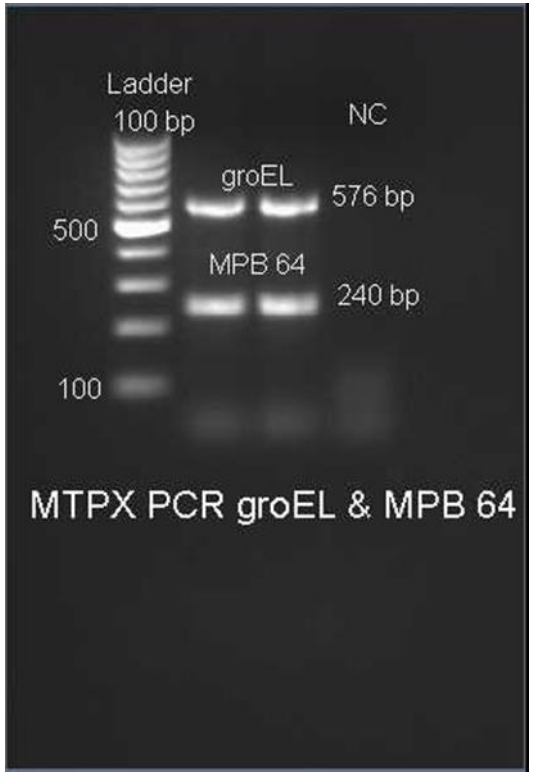
- Any body fluid
- An example for use in infertility testing
  - In investigation of infertility (sample endometrium)
  - Endometrium sheds in 4 weeks
  - Granuloma formation for HPE diagnosis takes 4 weeks
  - PCR enables diagnosis of HPE negative, Culture negative cases of TB endometritis
  - Enables early start of therapy
  - Sample of endometrium in late phase to be sent

MPB 64 gene

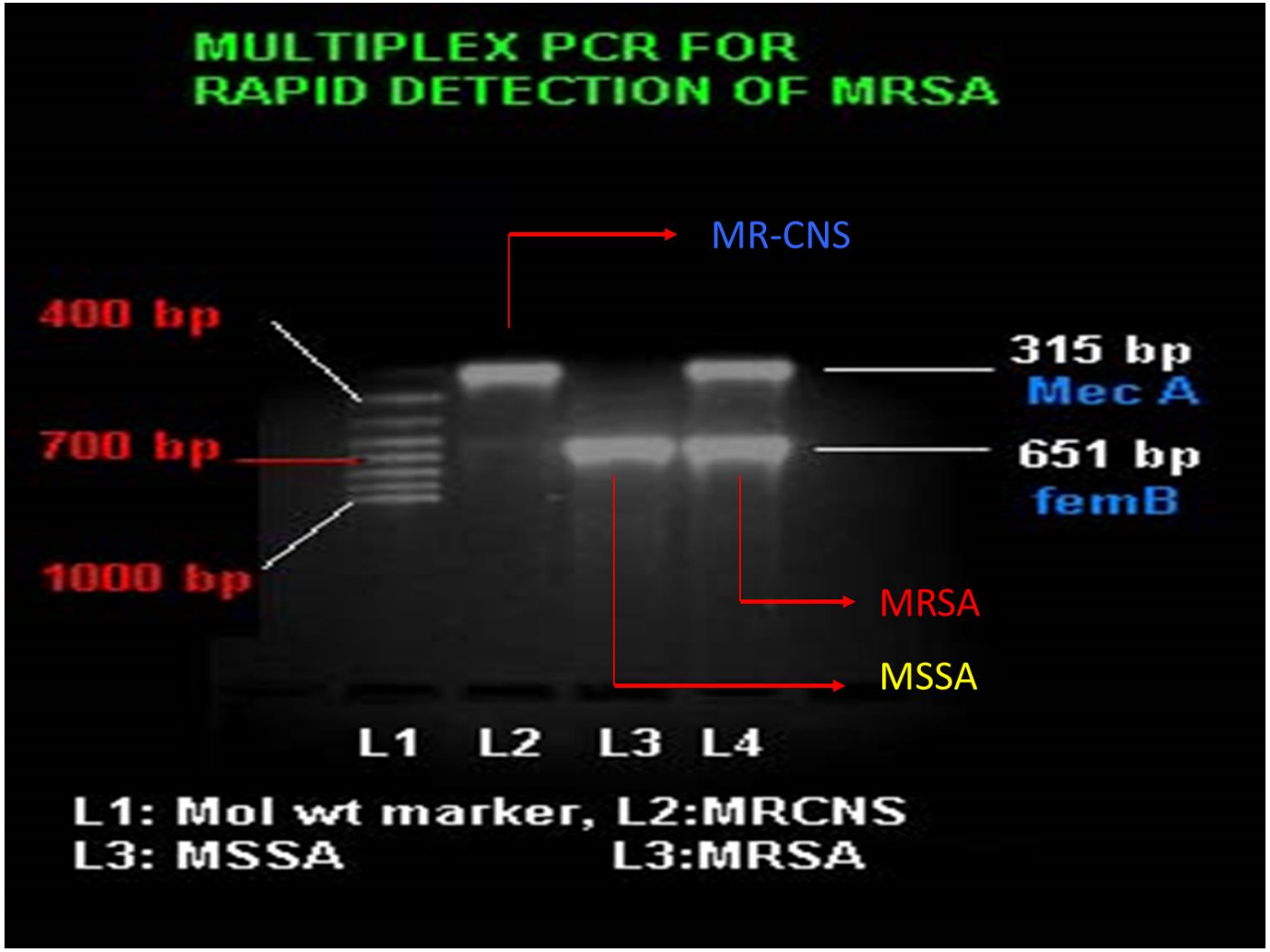


Two genes one Bacterium detection: TB PCR

- Using MULTIPLEX PCR
- Identify two Specific Mycobacterium Gene Coding Regions groEL & MPB 64
- Reduces False Positive Reports Significantly
- Highly Sensitive. Can Identify As Low As 2.3 CFU / Per PCR Reaction Mixture

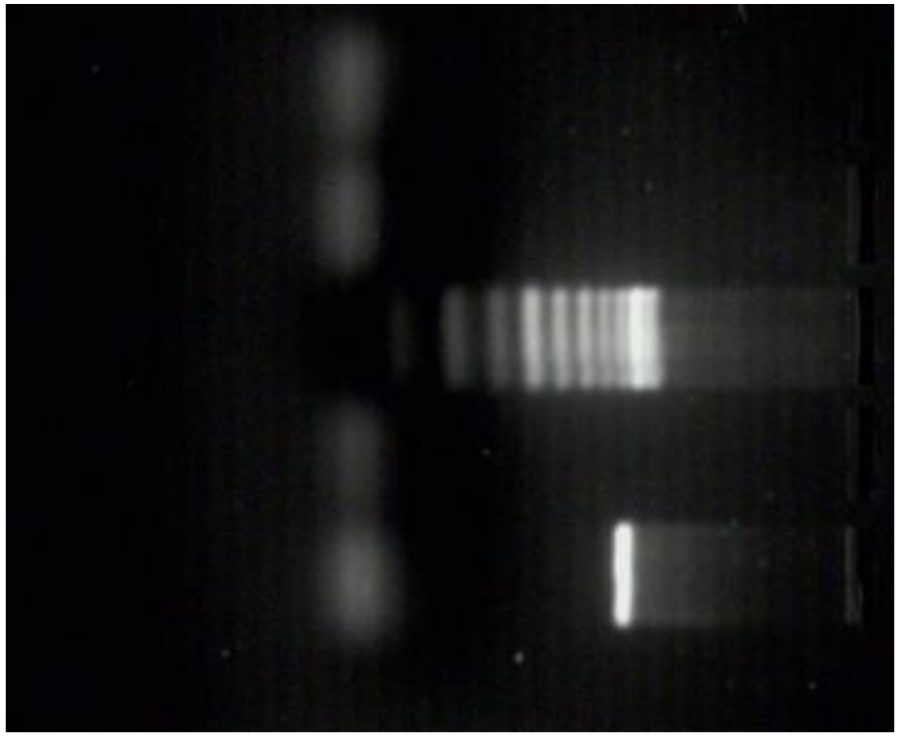


Two genes Two Bacterium detection: MRSA



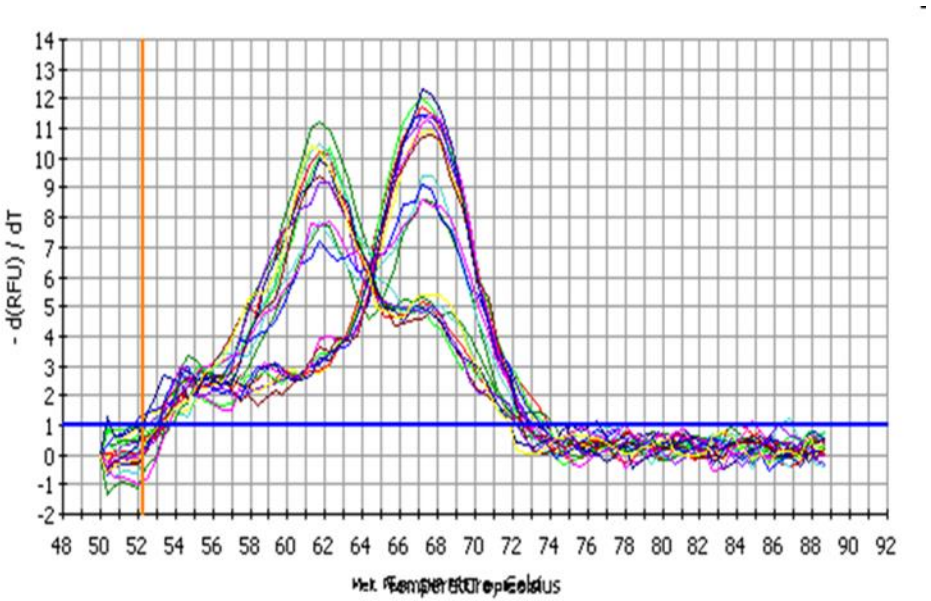
Transition to Real time PCR

PCR for RPO B gene



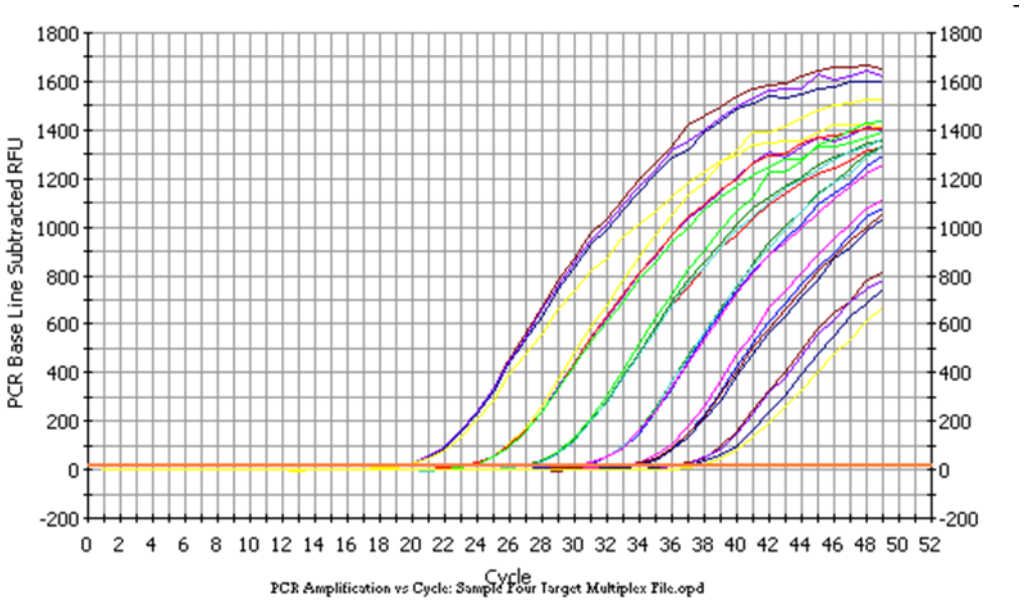
Melt curve analysis

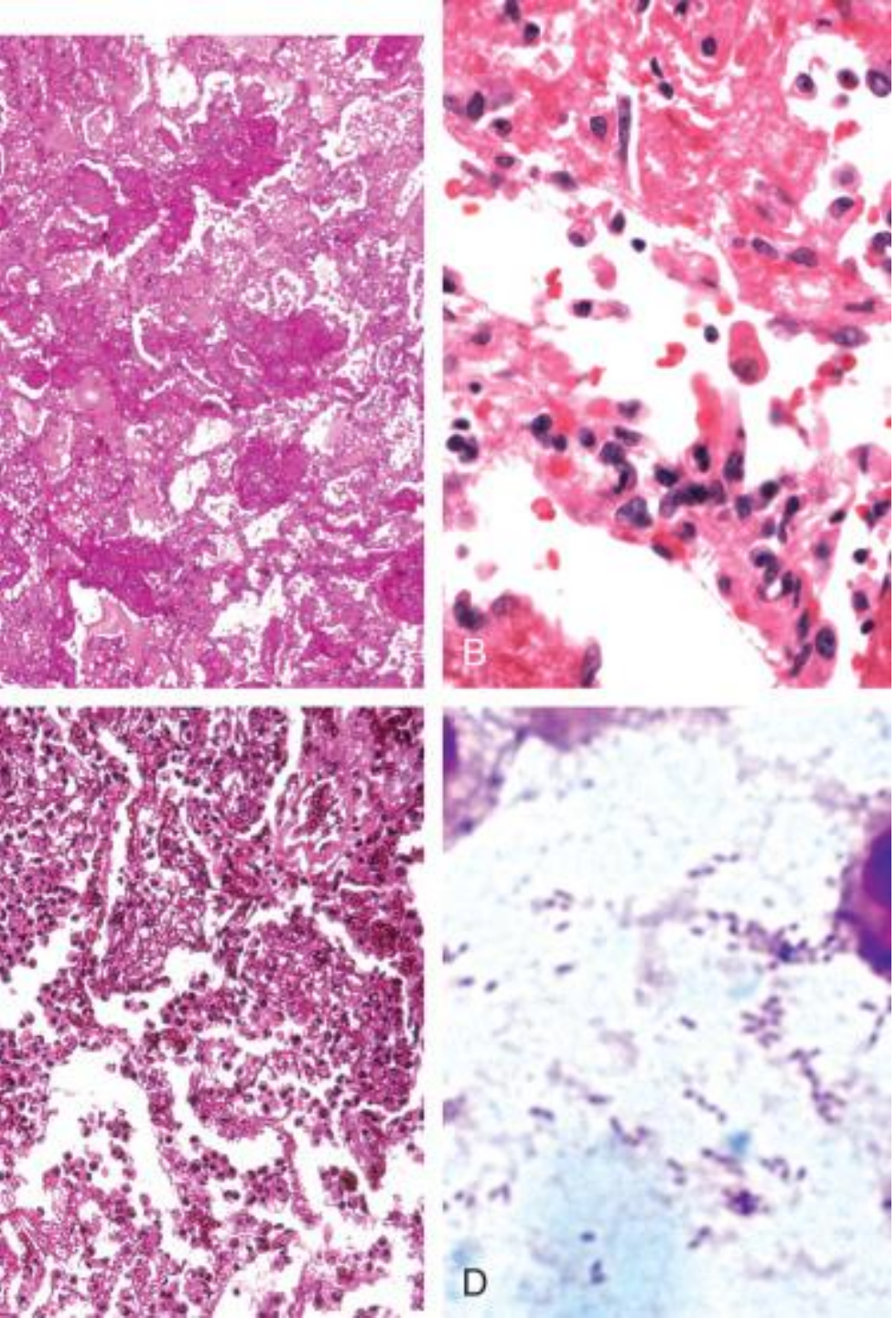
Early diagnosis of M.tuberculosis
Rifampicin resistance



Real Time PCR & Viral Quantification

- Real Time PCR
- Increased cycles sensitivity
- Probe specificity
- Quantification of organisms
- Ideal for syndromic approach





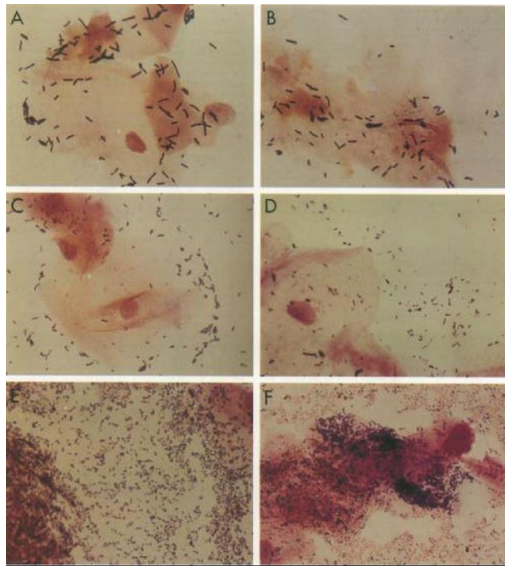
Usual syndrome panels target

- Respiratory Panel
- Blood Culture Identification Panel
- Gastrointestinal Panel
- Meningitis/Encephalitis Panel
- STI panel

Ghana Med J. 2016 Sep;50(3):142-148.

Bacterial etiology of sexually transmitted infections at a STI clinic in Ghana; use of multiplex real time PCR.

Sylverken AA[1], Owusu-Dabo E[2], Yar DD[3], Salifu SP[4], Awua-Boateng NY[3], Amuasi JH[3], Okyere PB[3], Agyarko-Poku T[5].



Real time PCR for syndromic approach

- Real time PCR based study
- Complementing the syndromic approach to STI management with pathogen detection
- 200 women tested
- 78.00% of the women were asymptomatic
- 77.1% of them tested positive for at least one bacterial STI pathogen.
- Mycoplasma genitalium was the most commonly (67.5%)
- Of those testing positive,
  - 25.0% had single infections,
  - 38.0% and 19.5% had double and triple infections respectively.
- Recognition that STIs in women are asymptomatic and regular empirical testing even for both symptomatic and asymptomatic patients is critical for complete clinical treatment.

Multiplexing RT PCR

Fast Track Diagnostics/Respiratory Pathogens 21 (Fast Track Diagnostics)"

Table 5: FTD Respiratory pathogens 21 - Possible Results

Master Mix	Pathogen	Signal in Green Channel	Signal in Yellow Channel	Signal in Orange Channel	Signal in Red Channel
FluRhino	IAV	POS	—	—	—
	HRV	—	POS	—	—
	IBV	—	—	POS	—
	IAV (H1N1) swl	—	—	—	POS
COR	HCoV 229	POS	—	—	—
	HCoV NL63	—	POS	—	—
	HCoV HKU1	—	—	POS	—
	HCoV OC43	—	—	—	POS
ParaEAV	HPIV-3	POS	—	—	—
	HPIV-2	—	POS	—	—
	HPIV-4	—	—	POS	—
	IC (EAV)	—	—	—	POS
BoMpPf1	HPIV-1	POS	—	—	—
	HMPV A and B	—	POS	—	—
	HBoV	—	—	POS	—
	M. pneumoniae	—	—	—	POS
RsEPA	HRSV A and B	POS	—	—	—
	HPeV	—	POS	—	—
	EV	—	—	POS	—
	HAdV	—	—	—	POS

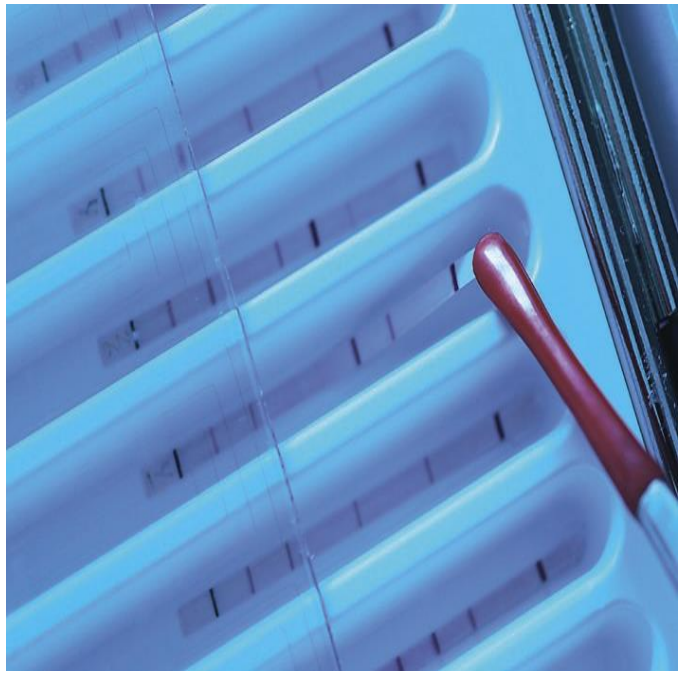
J Med Virol. 2019 May;91(5):731-737. doi: 10.1002/jmv.25379. Epub 2019 Jan 3.

Viral respiratory infections diagnosed by multiplex polymerase chain reaction in pediatric patients.

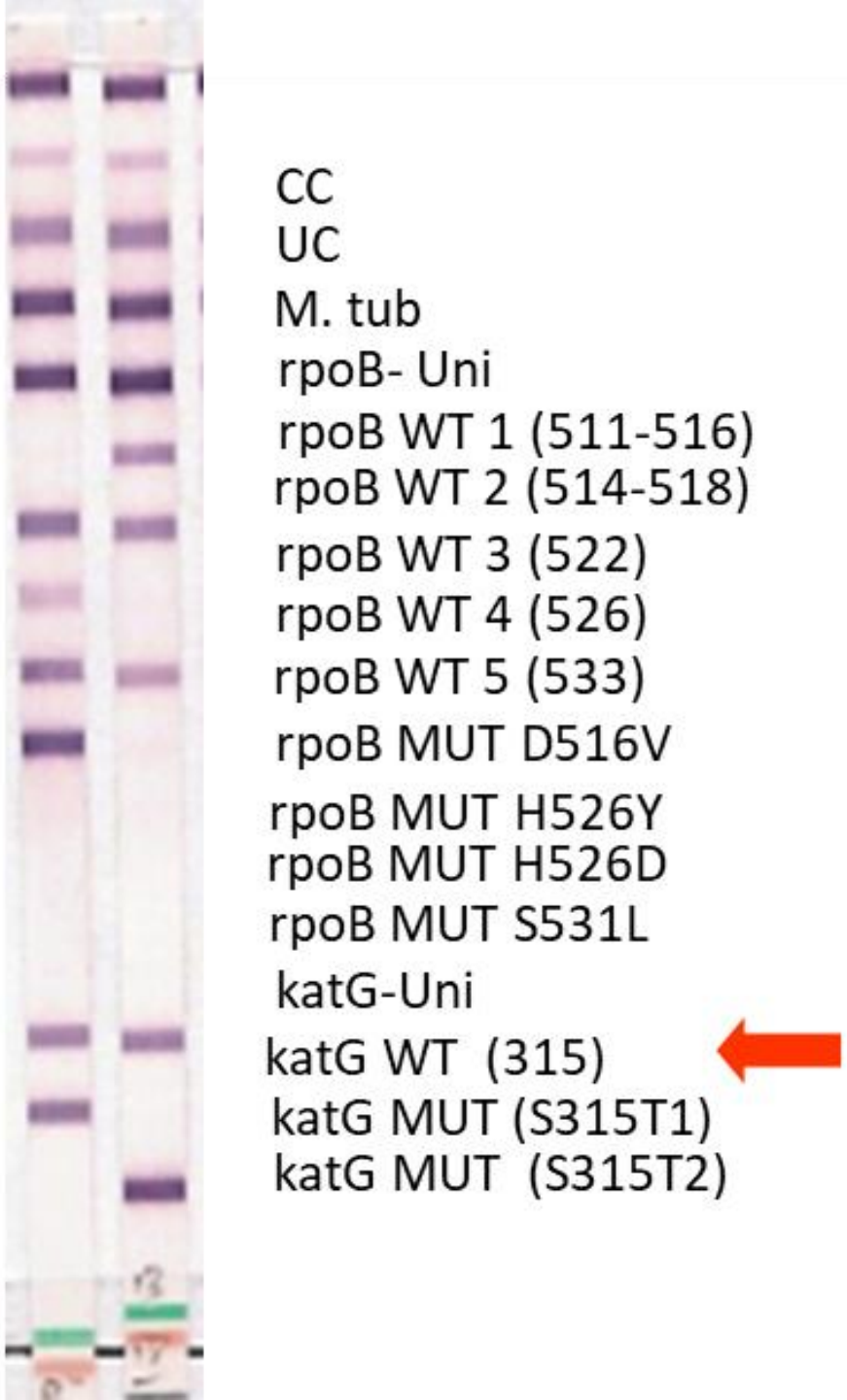
Appak Ö[1], Duman M[2], Belet N[3], Sayiner AA[1].

- A total of 3162 respiratory samples collected from children
- tested by two commercial multiplex real-time PCR assays
- Respiratory pathogens detected in 1857 of the 3162 (58.7%)
- The most prevalent viruses during the 8-year period were
  - rhinovirus/enterovirus (RV/EV; 36.2%),
  - respiratory syncytial virus (RSV; 19%),
  - influenza virus A/B (14.7%).
  - RV/EV and adenoviruses detected throughout the year.
  - Influenza virus was common during January to March
  - RSV and metapneumovirus were also seen in the same season
  - The coinfection percentage was 10.2%.

PCR Amplification and Hybridization to Membrane fixed Probes



- Probes hybridized to membranes/ strips
- Labelled PCR amplicons hybridized
- Colours developed
- Patterns read
  - Mdr Tb Testing: The GenoType® product series is based on DNA•Strip® Technology



PCR Amplification and Hybridization to Membrane fixed Probes

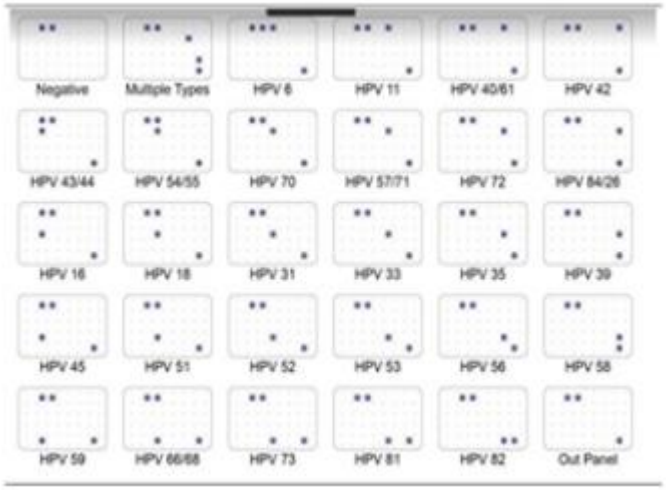
- Molecular genetic assay for Identification to Resistance to Rifampicin and/or Isoniazid
- ← Isoniazid-sensitive M. tuberculosis strain
- The wildtype probes show positive signal
- Both mutation probes are negative

Membrane Hybridization for Genotyping: HPV

Prominent findings

- 90 cases positive out of 220
- 25 different genotypes
- 56 had mixed infections

Citation: Kamel GG, Kumar M, Menon PK, et al. Molecular evaluation of common HPV genotypes for the patients attending thumbay hospitals, UAE. *Int J Mol Biol Open Access*. 2018;3(1):1-3. DOI: 10.15406/ijmboa.2018.03.00040



HPV genotypes	% Cases
66/68	17%
51	16%
6	15%
16	11%
43/44	6%
33	5%
52	4%
31, 33, 53, 42, 31, 56	3% each
18, 11, 45	2%
Others (26/84, 35, 39, 40/61,54/55, 57/71,	1% each

Number of viruses co infecting	Number of cases	%cases
1	34	38%
2	19	21%
3	13	14%
4	14	16%
5	6	7%
6	2	2%
7	1	1%
10	1	1%
Total	90	100%

Membrane Hybridization Syndromic Approach

J Clin Microbiol. 2018 May 25;56(6). pii: e01945-17. doi: 10.1128/JCM.01945-17. Print 2018 Jun.

Multicenter Evaluation of BioFire FilmArray Respiratory Panel 2 for Detection of Viruses and Bacteria in Nasopharyngeal Swab Samples.

Leber AL[1], Everhart K[2], Daly JA[3], Hopper A[3], Harrington A[4], Schreckenberger P[4], McKinley K[4], Jones M[5], Holmberg K[5], Kensinger B[5].

FilmArray® Respiratory Panel

Viruses

Adenovirus
Coronavirus HKU1
Coronavirus NL63
Coronavirus 229E
Coronavirus OC43
Human Metapneumovirus
Human Rhinovirus/Enterovirus
Influenza A
Influenza A/H1
Influenza A/H3
Influenza A/H1-2009
Influenza B
Parainfluenza 1
Parainfluenza 2
Parainfluenza 3
Parainfluenza 4
Respiratory Syncytial Virus

Bacteria

Bordetella pertussis
Chlamydophila pneumoniae
Mycoplasma pneumoniae

Overall 95% Sensitivity and 99% Specificity[1]

- A multicenter evaluation of 1,612 prospectively collected NPS samples
- The overall percent agreement between the FilmArray RP2 and the comparator testing was 99.2%.
- Positive percent agreement of 91.7%
- A negative percent agreement of ≥93.8% for all analytes.

Generations of DNA sequencing techniques

First Generation

- The first method of sequencing introduced in 1977 by Frederick Sanger.

Second Generation

- Massively Parallel sequencing

Third Generation

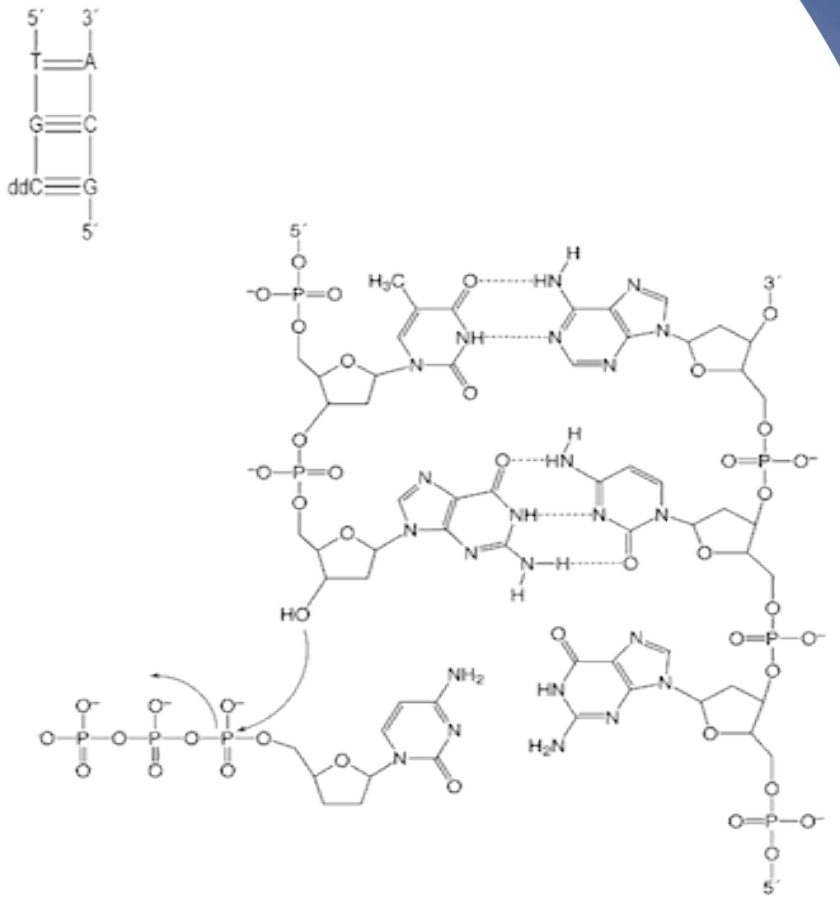
- Single molecule Sequencing.
- Includes Pac Bio “SMRT” (Single-Molecule sequencing in Real Time) in 2011 / Helicos tSMS.

Fourth (?) Generation

- Long Reads Sequencing
- Nanopore Sequencer

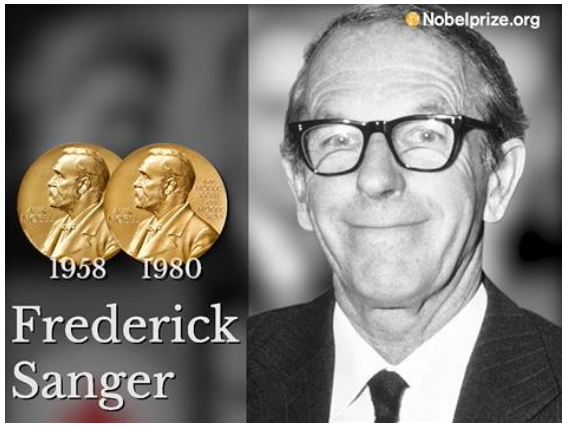
Sanger Sequencing

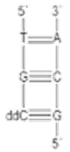
- The dideoxy method of DNA sequencing developed by Sanger et al. 1977
- DNA polymerases incorporate deoxynucleotide bases.
- The dideoxynucleotide lacks a 3'-hydroxyl group so further elongation of the chain is prevented
- Chain elongation is terminated selectively at A, C, G, or T.

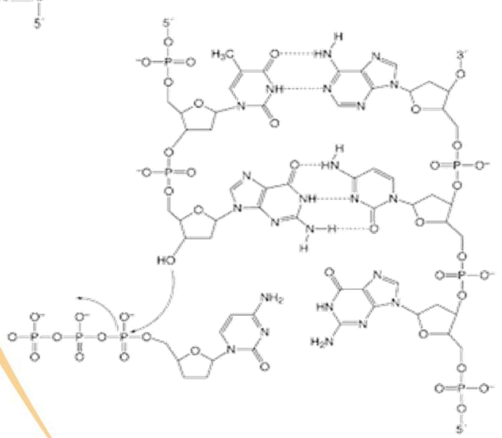


Fred Sanger

1918 to 2013







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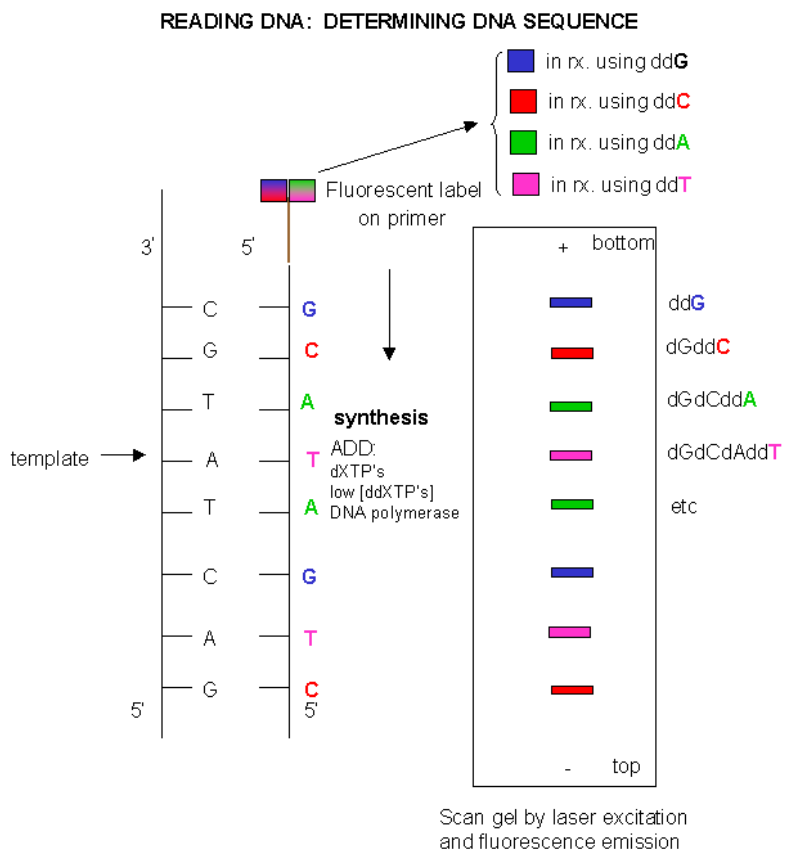
Sangers (contd)

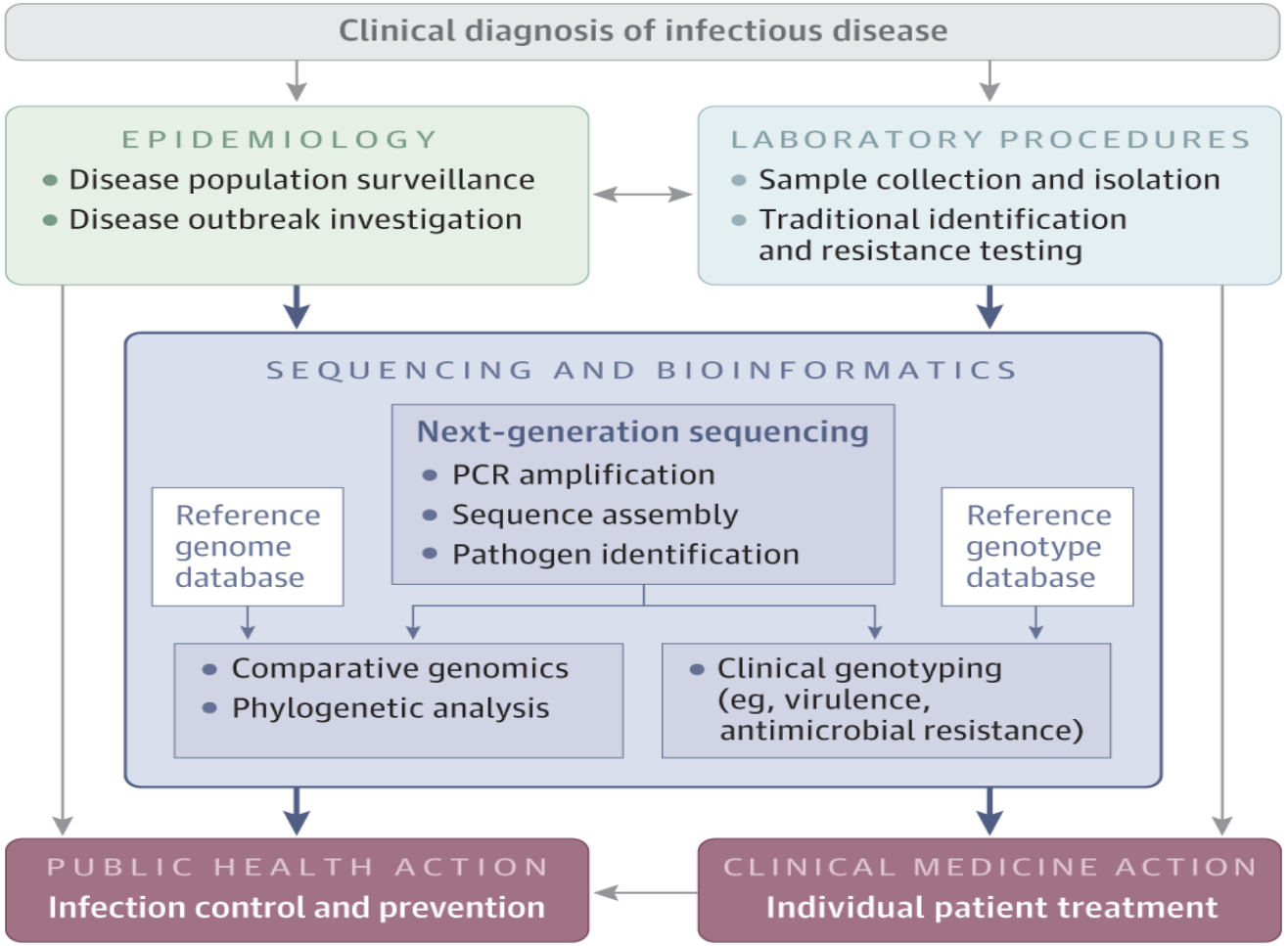
- First Gen Sequencing is primarily used for sequence detection, mutation analysis and genotyping in case of known sequences
- Not suitable for syndromic approach for disease diagnosis
- Second generation sequencers offer a good platform for syndromic approaches as well as quantitative metagenomics
- Third (? Fourth) generation sequencers may be the future of rapid infectious pathogen diagnosis by offering non amplified direct reads from isolated DNA



Sanger Sequencing

From: **Next-Generation Sequencing of Infectious Pathogens**

JAMA. 2019;321(9):893-894. doi:10.1001/jama.2018.21669



Workflow Transforming Pathogen Genome Sequence Data Into Actionable Information

Massively Parallel Sequencing (MPS)

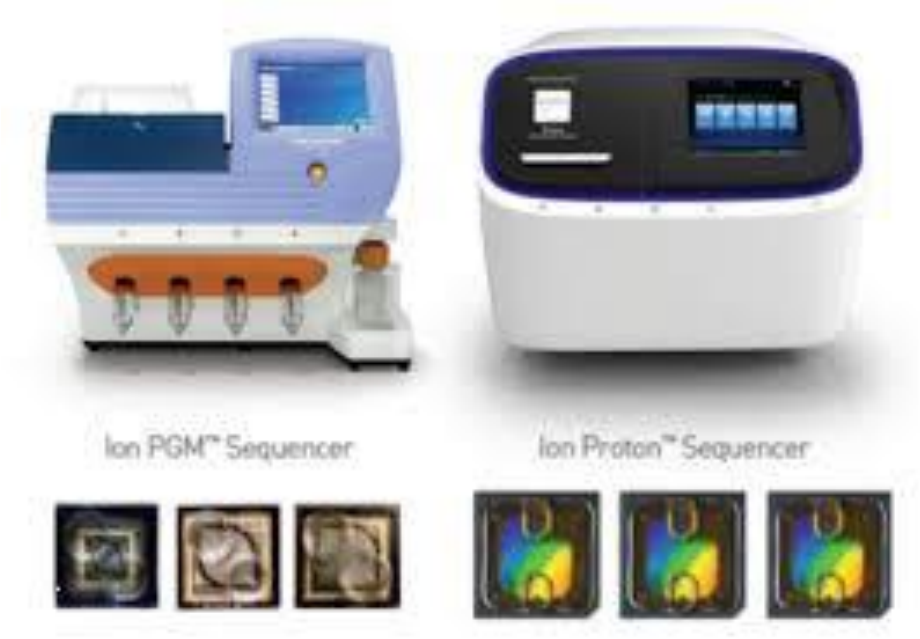
Principle of MPS:

- The concept behind MPS technology is similar to the Sanger method-
- DNA is cut into multiple small fragments
- DNA polymerase catalyzes the incorporation of fluorescently labeled deooxyribonucleotide triphosphate (dNTPs) in to a DNA template strand during sequential cycles of DNA synthesis.
- During each amplification cycle, incorporation of nucleotides is identified by different technologies.
- The data derived is analysed by computers by alignment of overlapping sequences

Types of MPS:

1. Illumina (Solexa) sequencing (Sequencing by synthesis).
2. Ion Torrent semiconductor sequencing.
3. 454 pyrosequencing (Roche 454 system).
4. SOLiD sequencing (Sequencing by ligation).

Two NGS technologies currently popular

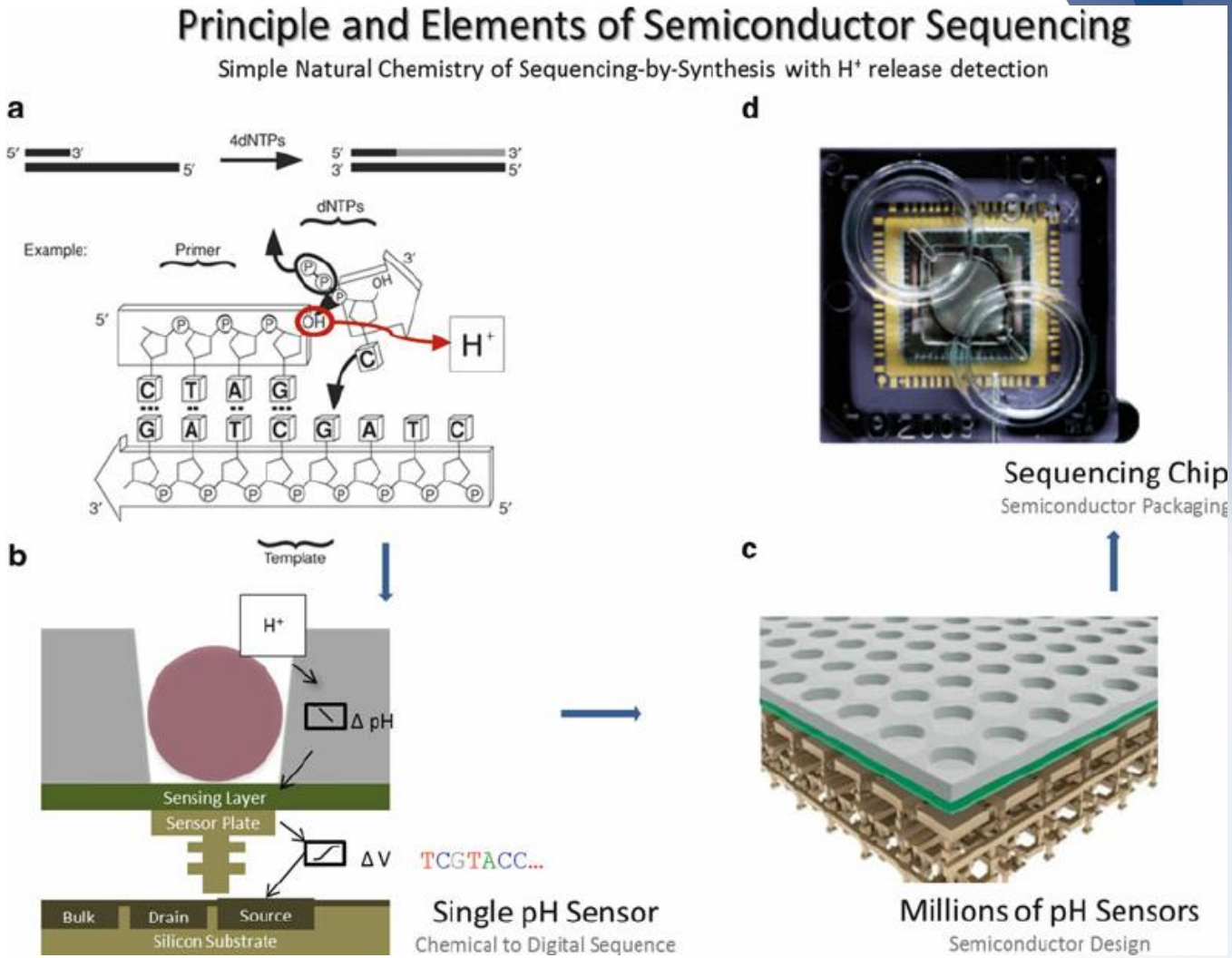


Ion Torrent semiconductor sequencer

Sequencing by semiconductor based sequencing

Ion Torrent Semiconductor Sequencing

- Ion Torrent was released at the end of 2010.[8,10]
- It uses semiconductor Chip.
- When a nucleotide is incorporated into the DNA molecules by the polymerase, a proton is released.
- By detecting the change in pH, PGM recognized whether the nucleotide is added or not.
- Each time the chip was flooded with one nucleotide after another.



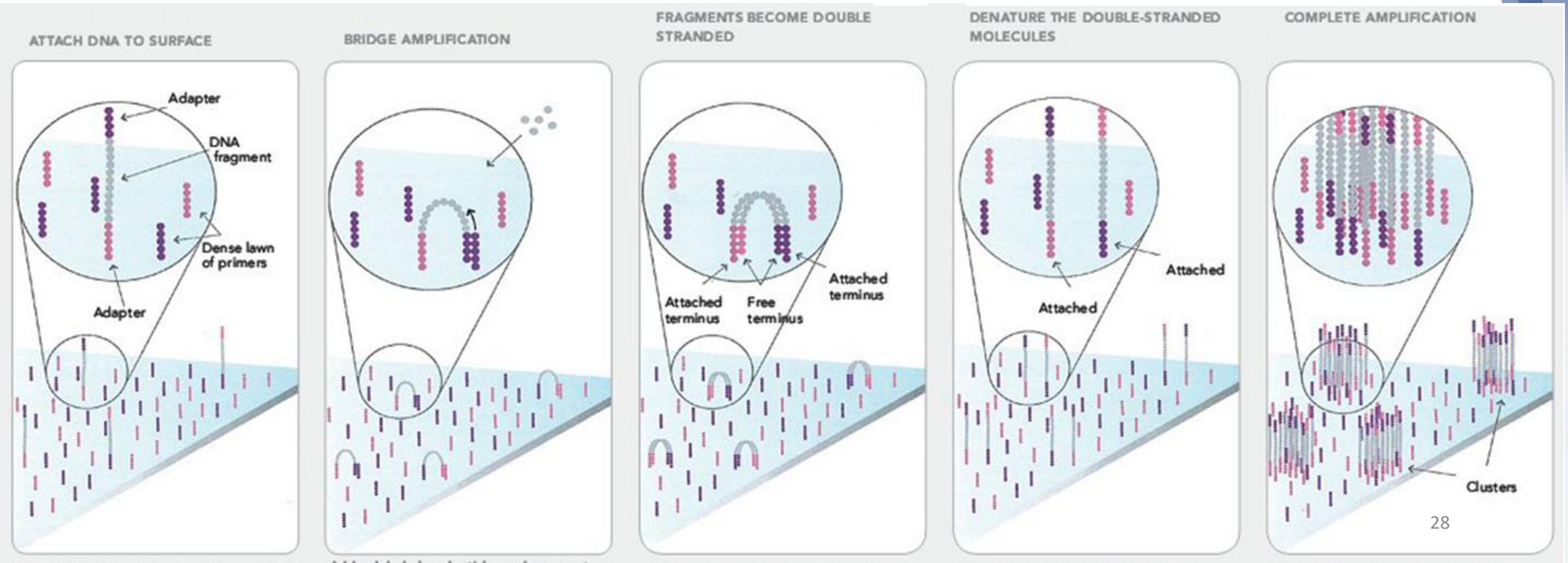
Two NGS technologies currently popular

Sequencing by synthesis

Illumina Genome analyzer

Reversible Terminator (HiSeq, MiSeq, NextSeq)

Cluster generation on a flow-cell surface



- Metagenomic sequencing can be used for detection of any pathogens using unbiased, shotgun next-generation sequencing (NGS), without the need for sequence-specific amplification.
- Proof-of-concept has been demonstrated in infectious disease outbreaks of unknown causes and in patients with suspected infections but negative results for conventional tests.
- Metagenomic NGS tests hold great promise to improve infectious disease diagnostics, especially in immunocompromised and critically ill patients.
- Examples from 2 separate validation studies are provided for steps from assay design, and validation of wet bench and bioinformatics protocols, to quality control and assurance.

Arch Pathol Lab Med. 2017 Jun;141(6):776-786. doi: 10.5858/arpa.2016-0539-RA. Epub 2017 Feb 7.

Validation of Metagenomic Next-Generation Sequencing Tests for Universal Pathogen Detection.

Schlaberg R[1], Chiu CY[1], Miller S[1], Procop GW[1], Weinstock G[1]; Professional Practice Committee and Committee on Laboratory Practices of the American Society for Microbiology[1]; Microbiology Resource Committee of the College of American Pathologists[1].

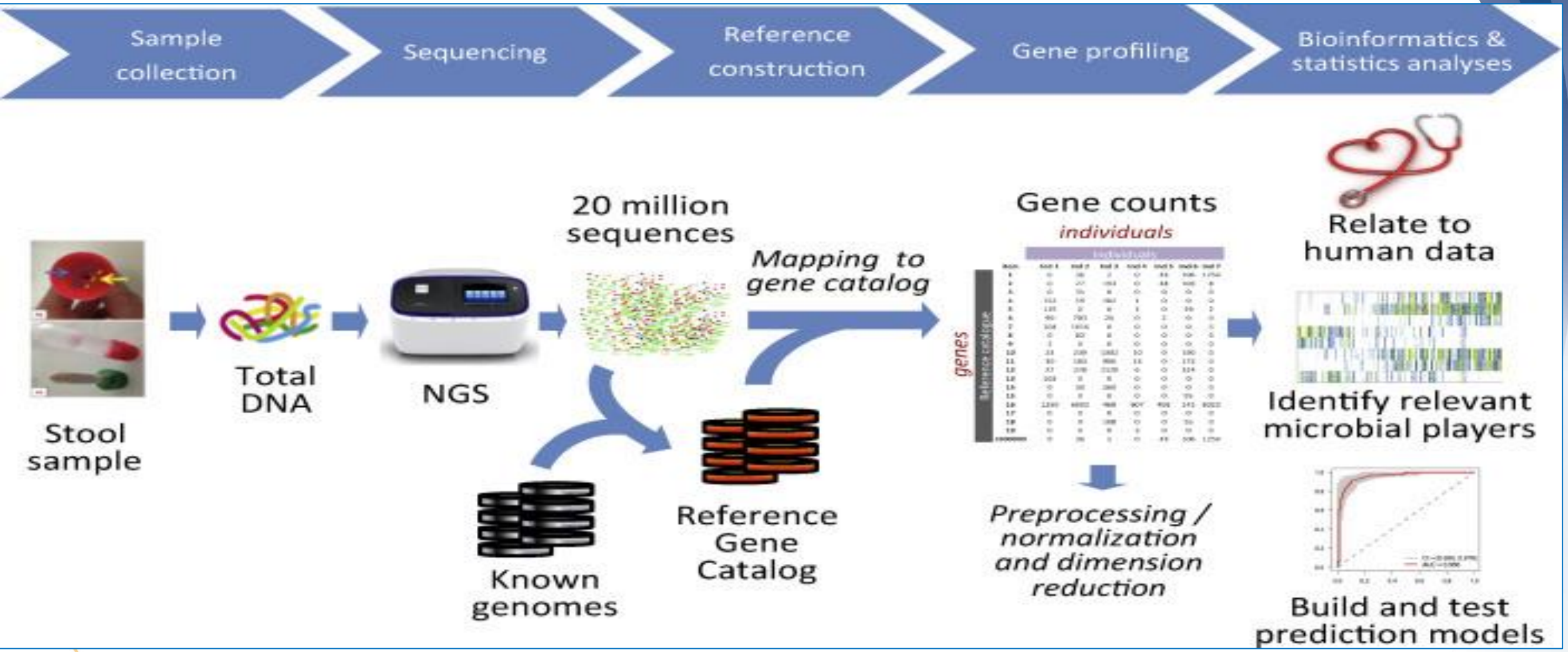
J Med Microbiol. 2019 Jul;68(7):996-1002. doi: 10.1099/jmm.0.000968. Epub 2019 May 28.

Detection of respiratory pathogens in clinical samples using metagenomic shotgun sequencing.

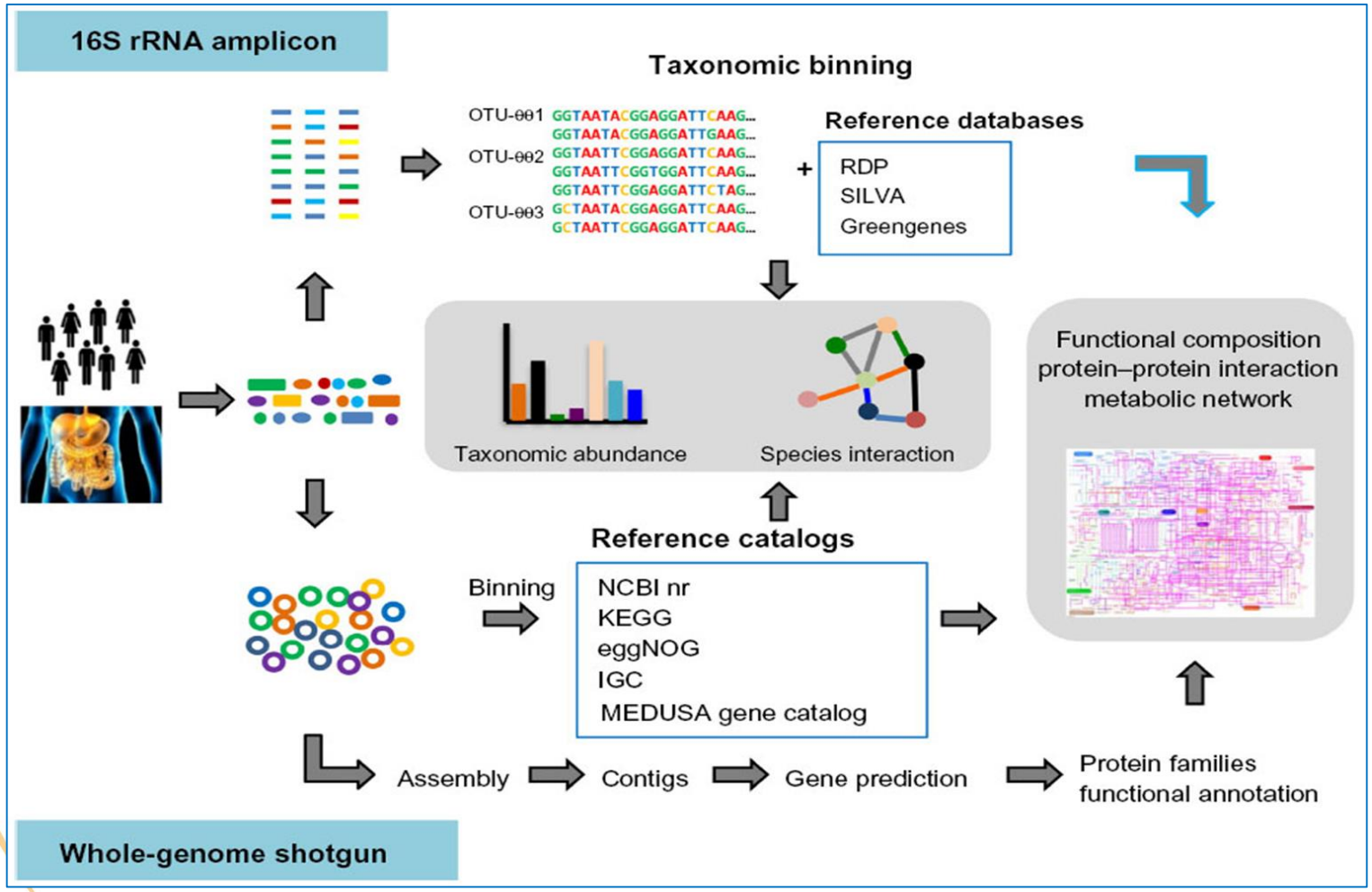
Qi C[1], Hountras P[2], Pickens CO[2], Walter JM[2], Kruser JM[2], Singer BD[2], Seed P[3,4,5], Green SJ[6], Wunderink RG[2].

- Shotgun metagenome sequencing (SMS) strategy on BAL samples from hospitalized patients with suspected VAP
- 67BAL samples from patients with VAP were tested
- SMS detected all pathogens recovered by cultivation approaches.
- In addition, putative pathogens other than the organisms recovered by culture were detected by SMS in culture-positive samples.
- In 40 of 45 (89 %) culture-negative samples, a potential pathogen was detected by SMS.
- SMS is able to detect bacterial, fungal and viral organisms in BAL, including culture-negative cases.

Quantitative metagenomics for the characterization of the human gut microbiome.

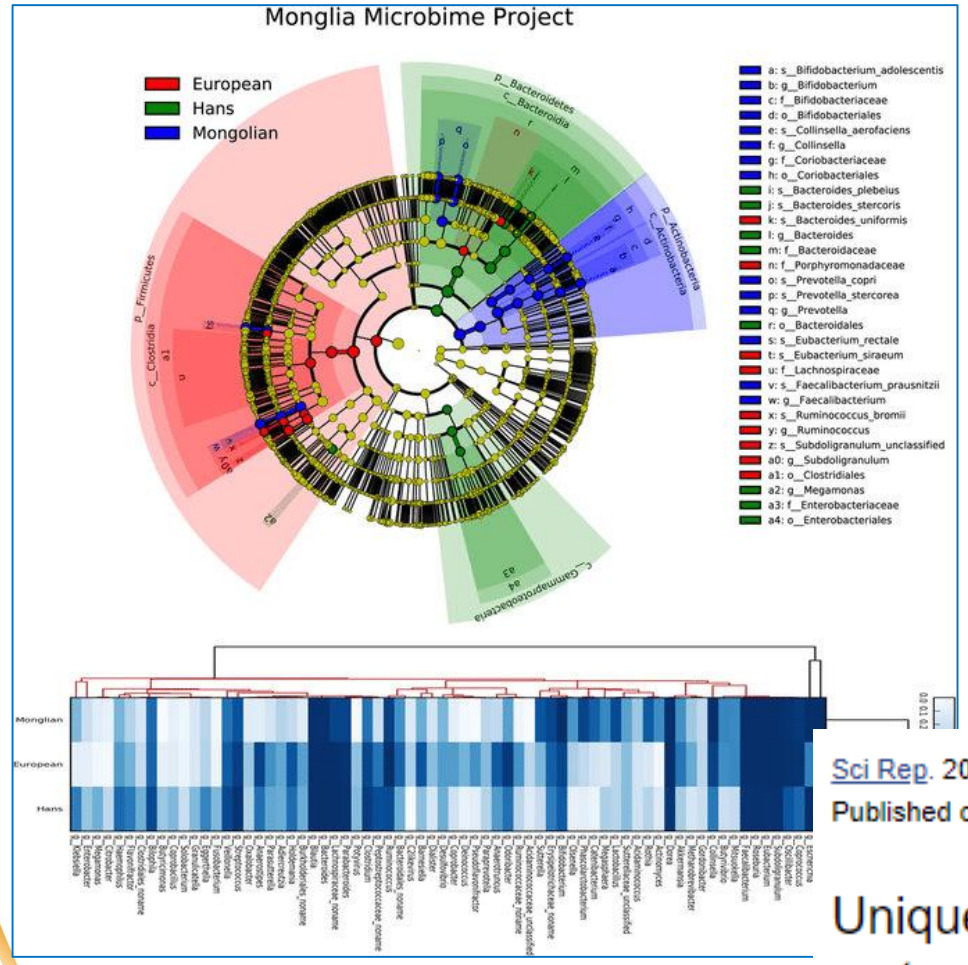


Bioinformatics for gut microbiota metagenomic analysis.



- The taxonomic assignment can be inferred by similarity-based or composition-based methods.
- With the taxonomic assignment of sequences, the species abundance can be characterized
- In the 16S rRNA-based approach, the function of the community can also be predicted using reference genome databases.

Gut Microbiome: Mongolia Microbiome Project



- 110 healthy Mongolian adults gut microbiota by shotgun metagenomic sequencing
- Data Compared with the intestinal microbiome among Mongolians, the Hans and European cohorts.
- Actinobaceria and *Bifidobacterium* were the key microbes contributing to the differences
- Metagenomic species analysis indicated that *Faecalibacterium prausnitzii* and *Coprococcus comes* were rich in Mongolian people
- The enriched genus *Collinsella*, a biomarker in symptomatic atherosclerosis might be associated with the high morbidity of cardiovascular and cerebrovascular diseases in Mongolian adults

Sci Rep. 2016; 6: 34826.
Published online 2016 Oct 6. doi: [10.1038/srep34826]
PMCID: PMC5052615
PMID: 27708392

Unique Features of Ethnic Mongolian Gut Microbiome revealed by metagenomic analysis

Wenjun Liu,[1,*] Jiachao Zhang,[1,*] Chunyan Wu,[2,*] Shunfeng Cai,[2] Weiqiang Huang,[1] Jing Chen,[2] Xiaoxia XI,[1] Zebin Liang,[2] Qiangchuan Hou,[1] Bing Zhou,[2] Nan Qin,[a,3] and Heping Zhang[b,1]

Author information ▶ Article notes ▶ Copyright and License information ▶ Disclaimer

OMICS. 2019 Oct;23(10):477-485. doi: 10.1089/omi.2019.0063.

New Insights on Obesity and Diabetes from Gut Microbiome Alterations in Egyptian Adults.

Salah M[1], Azab M[2], Ramadan A[3], Hanora A[2].

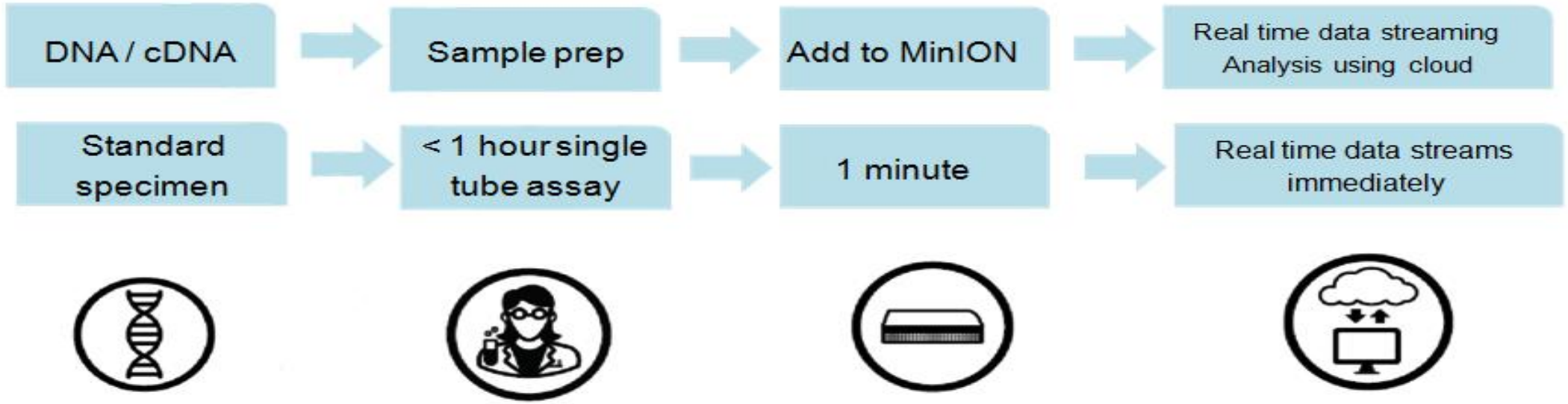
- Examined differences in gut microbiome in Egyptian adults from Egypt.
- Four study groups:
    1. Controls (C) with a normal body mass index, without obesity or diabetes,
    2. Obese adults (O) without diabetes,
    3. Adults with diabetes (D) who are not obese, and
    4. Adults who are both obese and diabetic (OD).
- 16S ribosomal RNA (rRNA) gene sequenced using the Illumina MiSeq platform.
- Ratio of Firmicutes/Bacteroidetes (F/B) displayed a remarkable increase in (OD) than controls.
- Faecalibacterium ($p < 0.05$) and Akkermansia ($p < 0.001$) distinguished (O) from controls,
- Fusobacterium ($p < 0.001$) and Bacteroides ($p < 0.001$) was significantly more abundant in (OD) compared with D.
- Obesity and diabetes were associated with remarkably enriched populations of Firmicutes and Bacteroidetes.
- The abundance of Fusobacterium is worth further research and exploration as a candidate biomarker for prediabetes especially in obese individuals.
- The potential antihyperglycemic activity of the gut microbiota is also noteworthy for future studies in other world populations.

From the First to the Fourth gen DNA sequencers.

- Church et al. and Deamer and Akeson separately proposed that it is possible to sequence DNA using nanopore sensors.

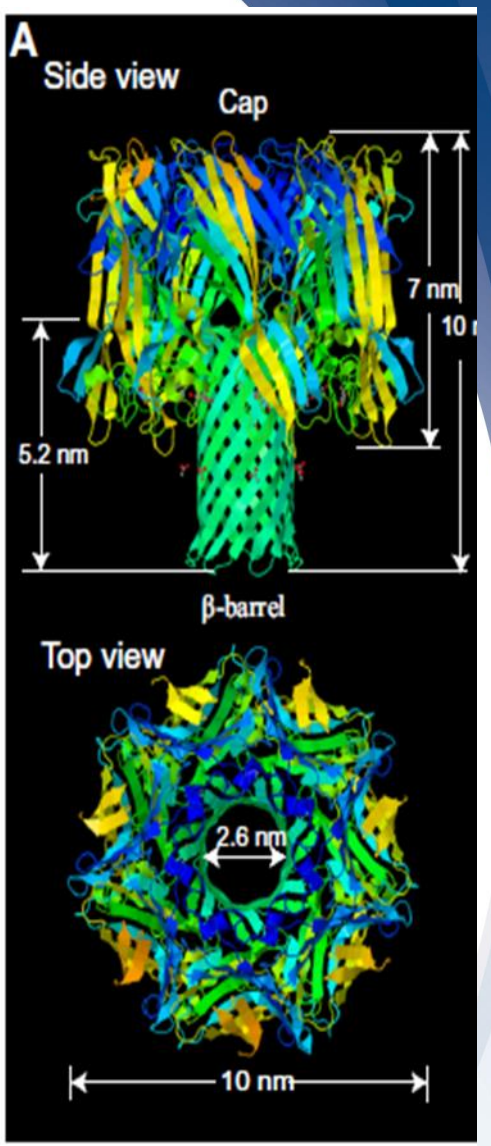
- The theory behind nanopore sequencing is that with the application of an external voltage, particles with sizes slightly smaller than the pore size are passed through the pore.
- Oxford nanopore technologies has miniaturized the commercial nanopore sequencing instruments to develop a MinION, first launched in 2013.
- The MinION is a single use device with the size of a USB memory stick and is designed for general applications of DNA sequencing.

MinION Workflow



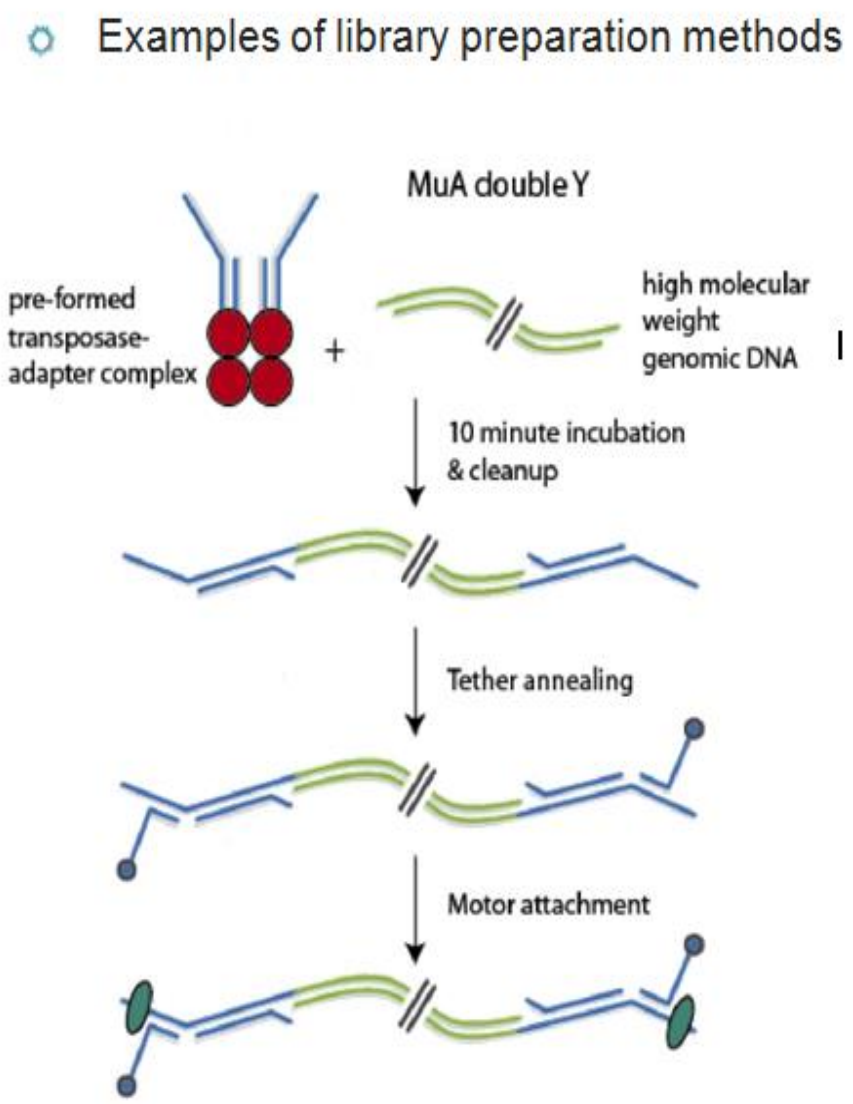
METRICHOR

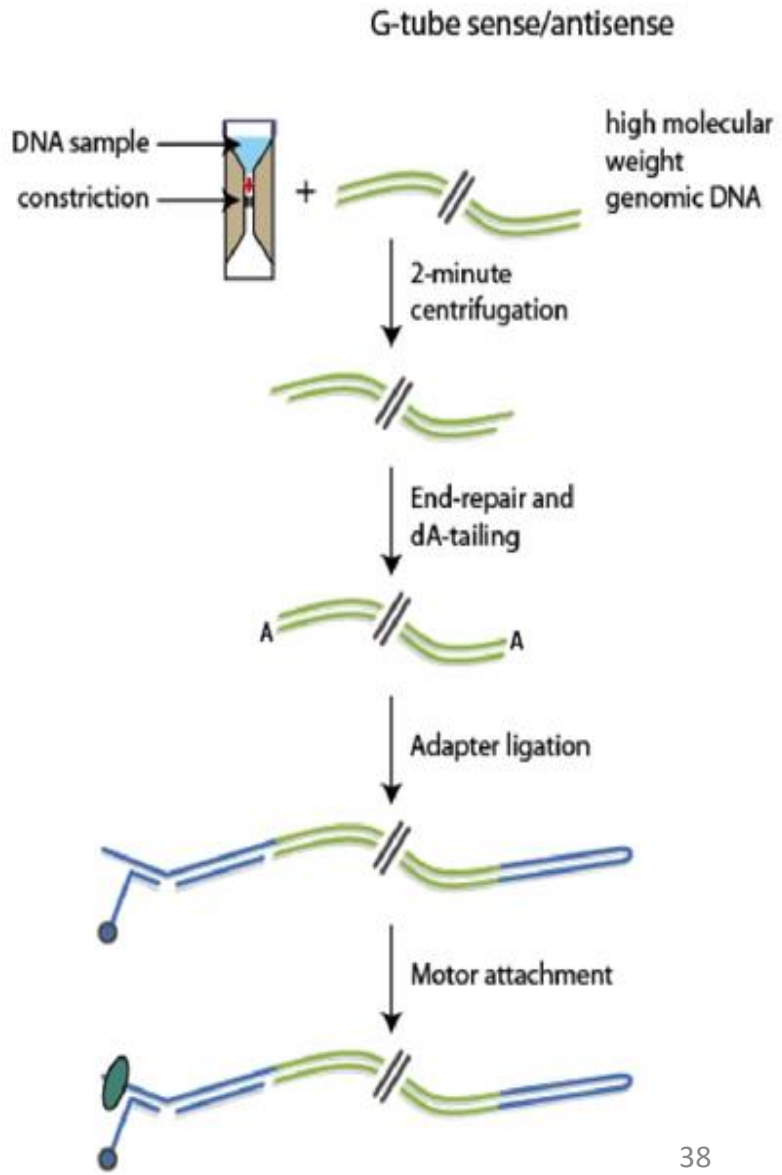
MinION Flowcell



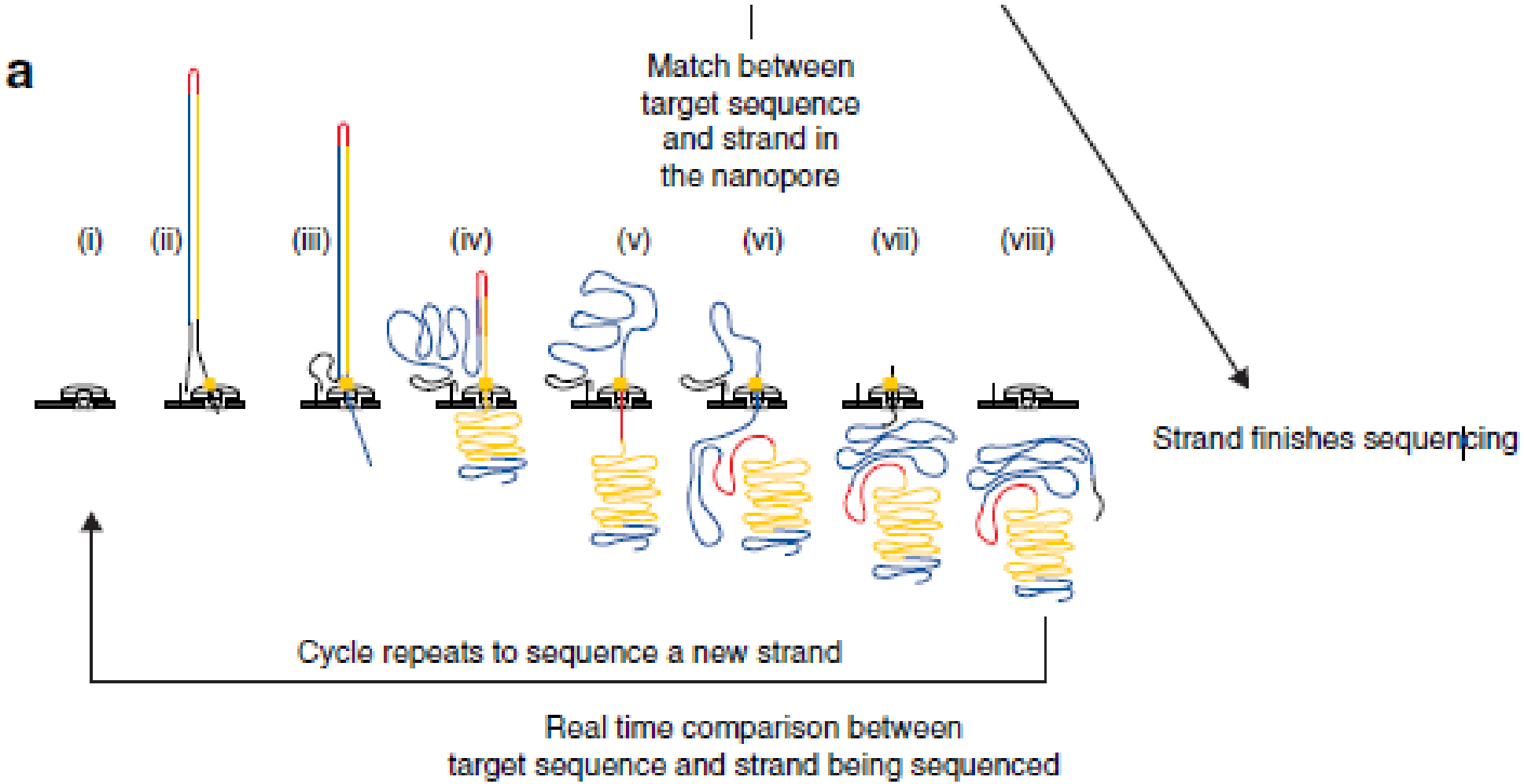
Library Preparation by ONT Kits

- Examples of library preparation methods





The process of Nanopore Sequencing



Nanopore sequencing

Nanopore (Fourth Generation)	First, Second and Third Generation
Ultra long reads (10^4 - 10^6) bases	Short read lengths
High throughput	Low throughput
Low cost	High cost
Low material requirement	High material requirement
Less complicated sample preparation	Complicated sample preparation and algorithms for data processing
Rapid DNA sequencing technology	

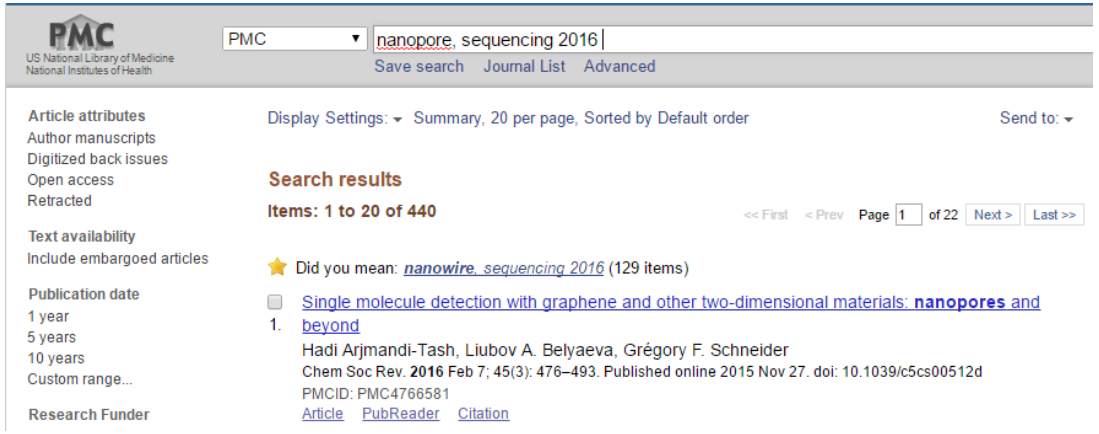
Applications

- 440 publications in 2016
- 1328 publications in 2019

- Laver T et al. in February 2015 assessed the performance of the Oxford Nanopore Technologies MinION by re-sequencing three bacterial genome from ATCC for *Streptomyces avermitilis (ATCC 35210), Borrelia burgdorferi (ATCC 31267) and Escherichia coli (ATCC 10798).* (7)

- *"We found that this device, which is the size of a USB stick, could detect the bacteria in heavily infected urine and provide its DNA sequence in just 12 hours. This is quarter of the time needed for conventional microbiology*."-Dr. Justin O'Grady from UEA's Norwich Medical School.

Emerging Technology

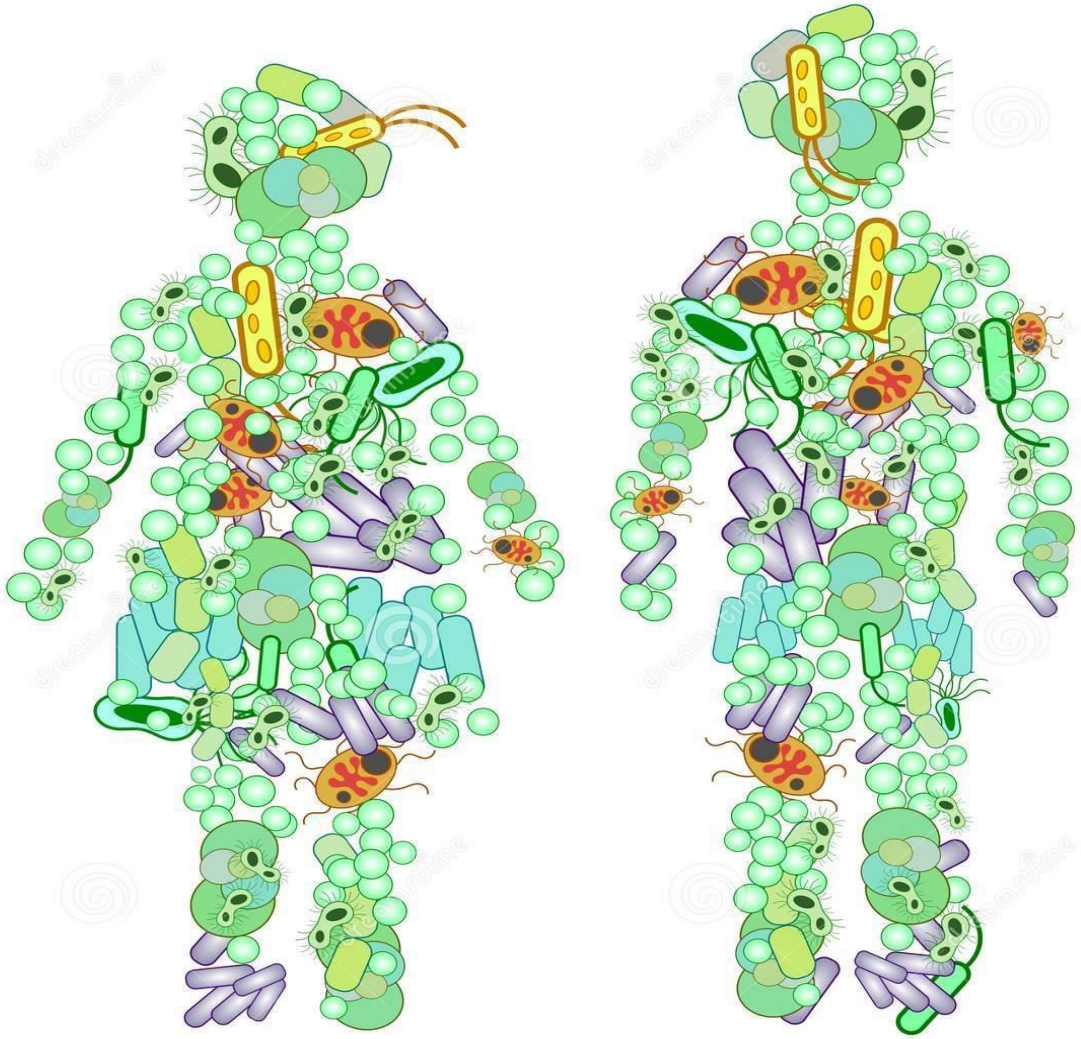


Applications

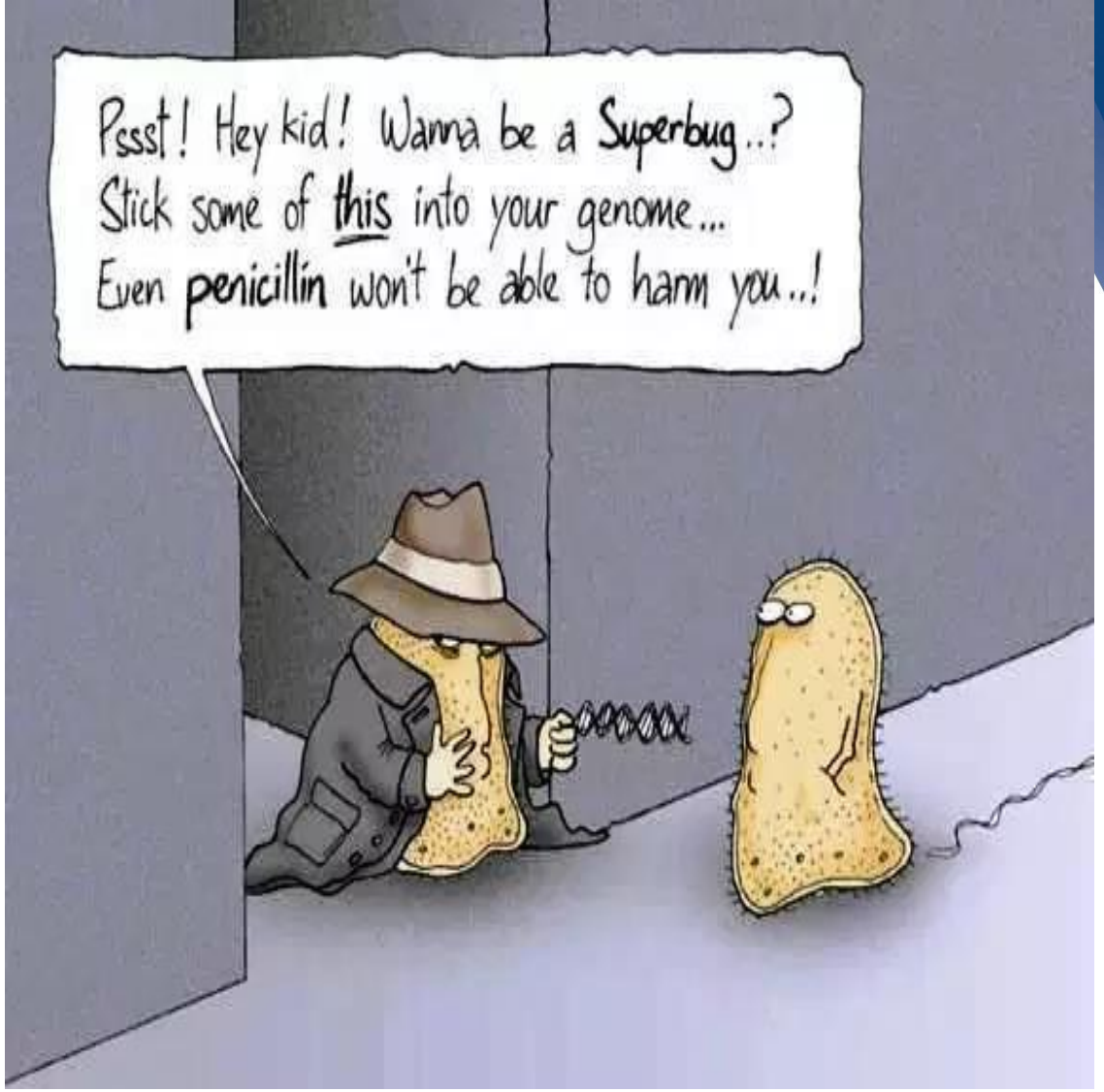
- Detection of base modifications
- Real-time targeted sequencing
- Direct RNA sequencing
- Bioinformatics and platform advances
- Single molecule protein sequencing
- Consensus sequencing for high accuracy
- Analysis of infectious agents at point-of-care
- Aneuploidy detection
- Nanopore detection in space

Conclusions

- Rapid evolution of technology
- PCR based procedures were used initially for rapid diagnosis of single pathogens
- Multiplex PCRs enabled us to look at multiple genes within the same bacterium and also distinguish genotypes
- Real time PCR helped truly multiplex and quantify pathogens
- Probe hybridization of amplicons was the next step to a syndromic approach
- DNA sequencing using NGS enabled us to identify the genotype of unknown pathogen without using pathogen specific primers
- NGS also helped in metagenomic analysis of mixed bacterial populations
- Long read sequencing now promises to be the future of pathogen identification by providing ultra short protocols for pathogen identification



Thank you


Pssst! Hey kid! Wanna be a Superbug..?
Stick some of this into your genome...
Even penicillin won't be able to harm you..!

بيورهيلث
purehealth

Thank you

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